Human Cobalophilin: The Structure of Bound Methylcobalamin and a Functional Role in Protecting Methylcobalamin from Photolysis†

Suzanne M. Frisbie‡,§ and Mark R. Chance*,‖

Department of Chemistry, Georgetown University, 37th and O Street Northwest, Washington, D.C. 20057, and Department of Physiology and Biophysics, Albert Einstein College of Medicine of Yeshiva University, 1300 Morris Park Avenue, Bronx, New York 10461

Received April 8, 1993; Revised Manuscript Received October 5, 1993®

ABSTRACT: The interactions of methylcobalamin with cobalophilin from human serum were analyzed using extended X-ray absorption fine structure (EXAFS) spectroscopy, photolysis of the cobalt–carbon bond of methylcobalamin, and a pK_a determination of the protonation of the coordinated nitrogen of 5,6-dimethylbenzimidazole (DMB). These results are consistent with the idea that the DMB nitrogen is still coordinated when protein is bound; however, the ability of a methyl radical (generated by photolysis) to escape the geminate cage of the protein is considerably reduced. For methylcobalamin in solution, the DMB nitrogen ligand is at a distance of 2.20 ± 0.03 Å from cobalt [Sagi, I., & Chance, M. R. (1992) *J. Am. Chem. Soc. 114*, 8061–8066]. This distance to the lower axial ligand does not change when protein binds (2.20 ± 0.04 Å), nor do the optical spectra exhibit any base-off character. The average of the distance from cobalt to the four equatorial nitrogens of the corrin plane is also unchanged. The pK_a for the conversion of the "base-on" to the "base-off" form of methylcobalamin, where the above DMB nitrogen becomes protonated and the Co–N axial bond is cleaved, does not deviate from the free cobalamin value of 2.7 when methylcobalamin is bound to cobalophilin. These results indicate that replacement of the DMB ligand with a ligand from the protein is unlikely. Although the background-subtracted EXAFS data sets for free methylcobalamin and for the protein complex are extremely similar, more accurate data with explicit higher shell analysis would be required to entirely rule out ligand replacement. The chemical and electronic nature of the ligand changes little. Photolysis of the photolabile methyl–cobalt bond shows a significant stabilizing effect by the protein reflected in a lowering of the quantum yield from 0.35 ± 0.04 for free methylcobalamin to 0.0055 ± 0.0004 for the cobalophilin–methylcobalamin complex. This effect may be physiologically significant in stabilizing methylcobalamin *in vivo* and indicates an important and previously unknown function for cobalophilin.

Human cobalophilin, a glycoprotein found in virtually every human biological fluid (Hippe et al., 1971), is present at a concentration of 10^{-10} M in plasma (Jacobsen & Huennekens, 1986). The plasma form is heavily glycosylated and differs from cobalophilin found elsewhere in its large sialic acid content (Nexo & Olesen, 1982). The protein portion of the molecule has an estimated molecular mass of 45 100 Da,[1] based on the cDNA sequence of the human gene (Johnston et al., 1989). The carbohydrate portion of the molecule accounts for the apparent molecular weight values of 121 000–138 000 obtained by gel filtration (Hall & Finkler, 1971). Apparent molecular weight values obtained by SDS–PAGE are in the range of 60–80 000 (Jacobsen et al., 1981).

The interactions of cobalamins with their transport proteins are not well understood. Of the three major cobalamin transport proteins found in human fluids (intrinsic factor, transcobalamin II, and cobalophilin), cobalophilin is the least specific and the tightest binder of the three (K_{assoc} = 10^{10}–10^{11} M^{-1}) (Schneider & Stroinski, 1987). It has become increasingly clear that the "nucleotide loop" portion of the cobalamin molecule is important for the binding of all three transport proteins. Recently, evidence has appeared that indicates that the ribazole (base and ribose) of cobalamin and the cobinamide portion are both needed for the binding of intrinsic factor to cobalamin (Andrews et al., 1991). In addition, Brown et al. (1988) have shown alterations in the NMR chemical shift value of the phosphate group of the nucleotide loop in a chicken cobalophilin–cobalamin complex.

It has been suggested that, upon binding of transport proteins to cobalamins, the bond between the bottom axial DMB ligand and the cobalt atom of the cobalamin is broken and replaced with a protein ligand. This hypothesis comes, in part, from a study of the UV–vis spectrum of cobalophilin bound to azidocobalamin, where γ2-bands indicative of a semireduced cobalt atom were observed (Nexo, 1978). However, the ^{31}P NMR data mentioned above show a significant downfield shift, which is opposite the trend expected for displacement of the DMB ligand (Brown et al., 1988). The trends in binding (specificity and affinity) for the various transport proteins are maintained when the metal to 5,6-dimethylbenzimidazole

† This research is based on work supported in part by the NRICGP-CSRS U.S. Department of Agriculture Program in Human Nutrients Grant No. 91-37200-6897. The construction and operation of beam line X-9A are supported by National Institutes of Health Grant RR-01633 and National Science Foundation Grant DMR-85190959. The National Synchrotron Light Source is supported by the Department of Energy, Division of Chemical Sciences.

* Author to whom correspondence should be addressed.

‡ Georgetown University.

§ Current address: National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bldg. 6, Room 109, National Institutes of Health, Bethesda, MD 20892.

‖ Albert Einstein College of Medicine of Yeshiva University.

® Abstract published in *Advance ACS Abstracts,* December 1, 1993.

[1] Abbreviations: Da, daltons; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EXAFS, extended X-ray absorption fine structure; Co(TPP), cobalt tetraphenylporphine; $Co(CN)_6$, cobalt hexacyanide; DMB, 5,6-dimethylbenzimidazole; k (wavevector) = $0.512336([E_{X\text{-ray}} - E_0]^{1/2}$, where $E_{X\text{-ray}}$ is the X-ray energy and E_0 is the threshold edge energy.

0006-2960/93/0432-13886$04.00/0

(DMB) coordination bond is changed by replacement of the cobalt atom with copper, zinc, and rhodium (Elsenhans & Rosenberg, 1984). The proteins preferentially bind to compounds where the DMB is presumed to be more strongly coordinated to the cobalt. Thus, the integrity of the Co–DMB bond is indicated to be important for binding.

Human cobalophilin's role as a B_{12} transporter is not absolutely required (Schneider & Stroinksi, 1987). One proposed function of cobalophilin involves its bacteriostatic effect (Nexo & Olesen, 1982), since cobalophilin is known to prevent the absorption of cobalamin by microorganisms (Gullberg, 1976; Gilbert, 1974). Increased levels of cobalophilin have been associated with many disease states and are used in the diagnosis of various metastatic tumors (Nexo & Olesen, 1982). There is only one binding site for cobalamins on human cobalophilin according to measurements of the increased Stokes radius of the protein when bound to radiolabeled [^{57}Co]hydroxycobalamin–albumin (Hippe et al., 1971) and according to direct binding studies with [^{57}Co]-cobalamin (Hippe & Olesen, 1971). Thus, EXAFS studies appear to probe a single class of binding site. The EXAFS studies of this article show minimal changes in cobalt–ligand bond distances upon binding of methylcobalamin to cobalophilin. Further evidence for the non-replacement of the DMB ligand by a protein ligand is that the pK_a of the base-on to base-off transition of methylcobalamin was found to be 2.7, regardless of cobalophilin binding.

MATERIALS AND METHODS

(*A*) *Purification of Human Cobalophilin.* The purification procedure is that used by Allen et al. (1974), with the following modifications: (1) vitamin B_{12} agarose was purchased from Sigma in lieu of synthesizing vitamin B_{12} Sepharose; (2) the affinity matrix was used in bulk form with decanting of the appropriate solutions (with approximately twice the volume of phosphate or guanidine buffer for each wash); (3) the renaturation of the protein was performed by addition of the buffer dropwise on ice; and (4) guanidine and excess cobalamin were removed via successive washing over a YM10 Amicon ultrafiltration membrane instead of dialysis. The reconstitution procedure employing guanidine hydrochloride for denaturation was used in order to easily insert methylcobalamin into cobalophilin. This is more difficult with other (photoaffinity matrix) purification schemes.

A 120-mL volume of vitamin B_{12} affinity matrix was purchased from Sigma in the form of cyanocorrin–agarose [vitamin B_{12}(acid hydrolyzed)–agarose] with 0.46 mg of cyanocorrin per milliliter of packed gel. The matrix was washed with distilled water until no unbound cyanocorrin was detected via UV–vis absorption. Approximately 12 L of human plasma was either purchased from the Washington D.C. Chapter of the American Red Cross or obtained as outdated plasma from the Georgetown University Hospital. The plasma was allowed to clot naturally at 4 °C for 2 h in 500-mL glass bottles. Cell debris, particulate matter, and clots were then removed by filtering 2× through glass wool and 2× through Whatman #1 filter paper. A 10-mL aliquot of washed cyanocorrin–agarose was added to each 500-mL lot of filtered serum. The beads were allowed to settle at 4 °C and then resuspended approximately every hour for 48 h to allow binding of cobalophilin to the matrix. After a final settling of the beads overnight, most of the plasma was removed by decanting. The beads were stirred gently and washed in distilled water several times (approximately 2400 mL total) until the absorbance of the wash at 280 nm was <0.1 absorbance unit. The beads were then washed 3× with 480 mL of glycine–NaCl buffer (0.1 M glycine, from Sigma, and 1.0 M NaCl, pH 7.5) and likewise with 0.1 M KH_2PO_4 (pH 7.5) to remove nonspecifically bound proteins. The beads were allowed to settle and the supernatant was discarded each time. The beads were then washed in 480 mL of buffer containing 0.1 M KH_2PO_4 and 5.0 M guanidine hydrochloride (at pH 7.5) five times for approximately 4 h each time, and the supernatant (which contains a variety of B_{12} binding proteins, but mainly cobalophilin and transcobalamin II) was retained. The guanidine releases bound protein from the beads by partially denaturing the protein. The same procedure was then applied to the beads using a buffer containing 0.1 M KH_2PO_4 and 7.5 M guanidine hydrochloride and finally using a buffer containing 7.5 M guanidine hydrochloride alone. For the last buffer, the beads were washed several times by suspending the beads at 4 °C, allowing them to settle, and then removing and collecting the supernatant. The beads were kept in the last buffer for at least 16 h total. The supernatant from this last buffer contained mostly cobalophilin with some transcobalamin II. Complete separation of cobalophilin from transcobalamin II can be achieved by lowering the ionic strength of the solution (using a buffer of 0.1 M NaCl and 0.1 M potassium phosphate (pH 7.5) for instance), ion exchange, or HPLC on a Rainen HIC-hydrophore column. In the present study, lowering the ionic strength of the protein solution was used to precipitate out the unwanted transcobalamin II, as outlined below.

To renature the cobalamin-free protein, 900 mL of cold glycine–NaCl buffer was added dropwise over 24 h at 4 °C to each 100-mL aliquot of denatured protein, to which methylcobalamin was added to a final concentration of 1 mM. As the ionic strength of the protein was lowered, transcobalamin II aggregated and precipitated. The slow renaturation was important in that it allowed for the ultimate recovery of substantial amounts of renatured cobalophilin without the transcobalamin II (the success of the separation of the proteins was determined via overloaded lanes on SDS–PAGE). The aggregates were removed by gentle filtration over a Whatman #1 filter. These 1-L solutions were then concentrated over a YM10 Amicon membrane to a final volume of 0.250 mL for each EXAFS sample, with a concentration of 300 μM or greater determined by the EXAFS edge jump of bound cobalamin. Unbound cobalamin and the remaining guanidine hydrochloride were removed by successive washes in 0.1 M KH_2PO_4 and 1 M NaCl over an Amicon YM10 membrane until no free cobalamin was observed in the wash. Great care was taken to ensure the removal of unbound cobalamin. Protein samples were run on 10% SDS–PAGE (Weber & Osborn, 1969; Davies & Stark, 1970), stained for glycprotein using Fuchsin–sulfite reagent from Sigma (Fairbanks et al., 1971), and then stained with Coomassie blue for protein (Weber & Osborn, 1969; Davies & Stark, 1970). A representative stained gel is shown in Figure 1. The cobalophilin comigrates with the BSA standard at approximately 66 000 Da. An overloaded gel (10× the amount) showed no other visible proteins. Also, the cobalophilin band stained pink with the glycoprotein-targeted reagent (not shown). Thus, this protein is identified as cobalophilin by B_{12} binding, molecular weight, and identification as a glycoprotein.

(*B*) *EXAFS Experiment.* EXAFS experiments were performed at the National Synchrotron Light Source of Brookhaven National Labs on beam line X9-A. A high count rate energy-resolving germanium detector with 13 elements

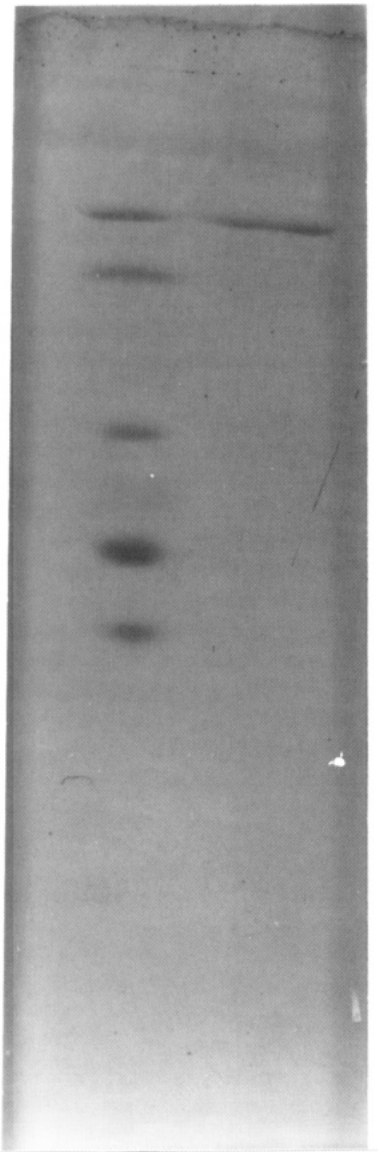

FIGURE 1: SDS–PAGE (10% gel) of purified cobalophilin (lane 2) compared to molecular weight standards (lane 1): 66 000, bovine plasma albumin; 45 000, ovalbumin; 34 700, porcine stomach mucosa pepsin; 24 000, PMSF-treated bovine pancreas trypsinogen; 18 400, bovine milk β-lactoglobulin. The cobalophilin comigrates with the bovine albumin marker.

was used for data collection (Cramer et al., 1988). Vertical slits were adjusted to give 3-eV resolution at the cobalt K-edge for EXAFS experiments (2 mm vertical) and adjusted to 0.75 mm vertical for edge experiments, which provides the maximum resolution available with the Si(111) crystals used (Chance et al., 1986a; Sagi et al., 1990). All experiments were performed using a Si(111) monochromator crystal (unfocused beam) and a liquid nitrogen cryostat (Powers et al., 1981), which kept the samples at approximately 160 K. Data were taken over four regions: from 150 eV below the edge (7.725 eV) to 25 eV below the edge with data taken at 10-eV steps and 2 s per point; from 25 eV below the edge to 30 eV above the edge using 2-eV steps and 1 s per point; from 30 eV above the edge to 450 eV above the edge using 3-eV steps and 4 s per point; and finally from 450 eV above the edge to 600 eV above the edge using 3-eV steps and 6 s per point. To avoid saturation (dead time) effects, the data were collected at relatively low (40 000 counts s^{-1}) internal count rates for each of the 13 channels.

In order to obtain the largest number of scans with adequate

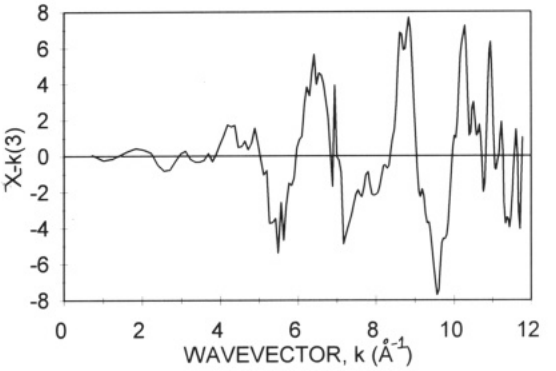

FIGURE 2: Background-subtracted, k^3-weighted EXAFS data of methylcobalamin bound to human cobalophilin. This data has been deglitched, and polynomials have been interpolated over the glitch regions.

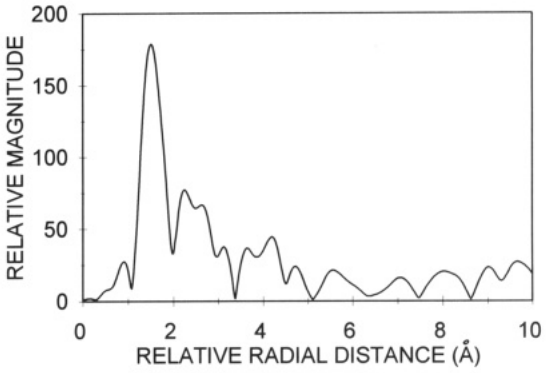

FIGURE 3: Fourier transform of Figure 2.

count rates from the smallest amount of sample, a special data collection scheme was devised. One sample was divided into eight horizontal subsections, and each subsection was scanned individually by altering beam slit positions. This allowed numerous scans on one sample that could be coadded, compared to only a few scans if the entire sample was exposed to the beam at one time. The vertical size of the beam was kept at 2 mm and the horizontal size was kept at 1.6 mm. After six scans on one sample area, sample damage was observed as a visible brown color on the sample. Therefore, only four scans or fewer were taken on each subsection, after which no sample damage was visible. Sample integrity was assured by UV–vis monitoring of samples both before and after a set of scans to find evidence of sample disintegration. The University of Washington EXAFS package on the Georgetown University 8700 VAX computer was used for data analysis (Sagi et al., 1990). Fifty-eight scans were collected that were usable, derived from samples available from three separate purifications. A cubic polynomial (b-spline) background (isolated atom) subtraction was performed with k^3 weighting (Figure 2) followed by Fourier transformation (Figure 3). The first EXAFS shell was windowed and then back-Fourier-transformed. The back-transform window was chosen by visual inspection of the Fourier-transformed data and was positioned at 0.7–2.1 Å on the radial coordinate, with cosine-squared tailing on each side to allow flexible isolation of the first shell. The background-subtracted data were also Fourier-transformed using adjustable cosine-squared tapered windows.

The Fourier-transformed data were analyzed by nonlinear least-squares fitting to model compounds (Figure 4; Lee et al., 1981). Co(TPP), which contains four nitrogens at a 1.949-Å average distance from cobalt (Madura & Scheidt, 1978), and $Co(CN)_6$, which has six carbons at 1.893 Å from

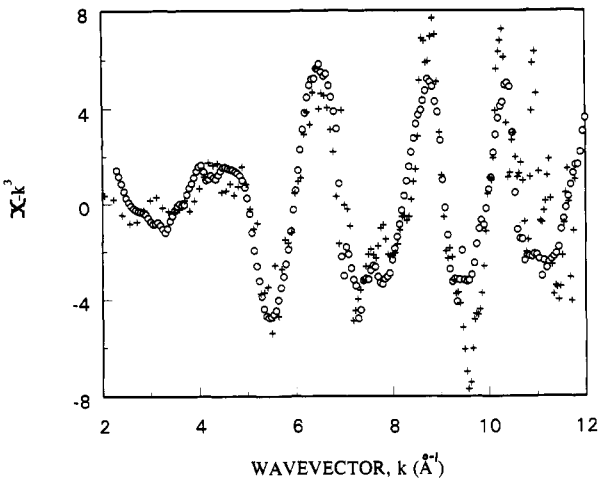

FIGURE 4: Background-subtracted, k^3-weighted EXAFS data of methylcobalamin (O) compared to data for the cofactor protein complex (+).

Table I: Nonlinear Least-Squares Fitting Solutions for Methylcobalamin Bound to Human Cobalophilin[a]

fit type	N	r (Å)	$\Delta\sigma^2$ (Å^2)	ΔE_0 (eV)	χ^2
one-atom fit	6	1.91 ● 0.06	0.006	–4	25.5
two-atom fit	4	1.90 ● 0.01	0.0004	–6	0.78
	2	2.13 ± 0.05	0.007	–2	
	5	1.90 ± 0.01	0.004	–4	1.04
	1	2.20 ± 0.03	–0.004	+2	
three-atom fit	4	1.90 Co–N	0.0004	0	0.71
	1	1.90 Co–C	–0.002	–2	
	1	2.20 Co–N	–0.002	+2	

[a] r is the distance from cobalt in Å, N is the coordination number, E_0 is the energy shift relative to the model compound, $\Delta\sigma^2$ is the Debye–Waller factor shift relative to the model compound, is the χ^2 sum of residuals squared. Fourier-transform windows were set at 1.5–12.0 Å^{-1} with 2.0 Å^{-1} cos^2 tapered windows, the back-transform window was 0.7–2.1 Å with 0.2 Å cos^2 tailing, and the fitting range was 4.0–12.0 Å^{-1}. The errors quoted in this table are based on mapping out the minimum solution with respect to χ^2; the total errors are quoted in the text.

cobalt (Kruger, 1978), were used as models. Fourier transform and filters for the models were identical to those of the unknowns for any particular reported fit. Error analysis and the acceptable range of edge energies and Debye–Waller factors have been described previously (Chance et al., 1986 a–c; Sagi et al., 1990; Sagi & Chance, 1992). Due to the relatively high noise level in the data, a number of precautions were taken to assure the adequacy of the results. First, several types of error analyses were performed. Partial sums of the 58 scans were analyzed independently and compared for the differences in fit distances. Also, the minimum well for each solution was mapped out, and the dependence of χ^2 on variations in individual parameters was examined. Noise levels, especially at high k values, can be influenced by the degree of tailing provided in the UW-EXAFS package. These tails were systematically varied and the results of the fitting analyzed. Lastly, the data were deglitched and polynomials were fit over short regions of the data that were especially noisy. These data were also analyzed. The reported errors encompass the full range of solutions that was found by the individual methods. In fact, the different analyses agreed within their respective errors (defined by mapping out the minimum χ^2). These error analysis methods are described in detail in Sagi et al. (1990), Sagi and Chance (1992), and Lytle et al. (1989). The results of the fitting in Table I are based on the un-deglitched data with the tailing discussed above. Figures 2 and 3 show deglitched data with minimal

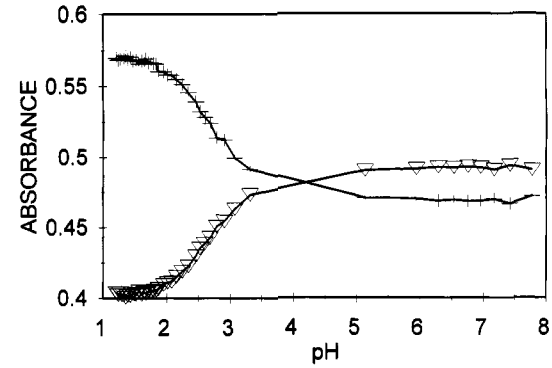

FIGURE 5: Titration of methylcobalamin–cobalophilin with 0.5 M HCl. Plot shows the titration results at two wavelengths: 520 nm, which records the decrease of the base-on species (a) (▽), and 460 nm (+), showing a rise due to the appearance of base-off methylcobalamin (b). Data show inflection points at pH values of 2.7 ± 0.1 (experimental details are in the text).

tailing. The important criterion is the noise in the filtered data.

(C) pK_a Determination of Methylcobalamin. Cobalophilin with a bound methylcobalamin concentration of approximately 0.07 mM was used for pK_a determinations. The protein was in a buffer of 0.1 M potassium phosphate and 1.0 M NaCl (pH 7.5) at the outset of the experiment. All UV–vis spectra were taken using a Hewlett-Packard 8452A diode array spectrometer with an integration time of 0.1 s and a starting sample volume of 2 mL. Absorbance was measured in a quartz cuvette of 1.0 cm path length which had been precoated with a solution of 0.1 mg mL^{-1} BSA. A 0.5 M HCl solution was used for titration purposes. UV–vis spectra were taken of the base-on to base-off protein-bound methylcobalamin conversion after each addition of acid. Spectra were corrected for the appropriate dilution volume of added acid and were also corrected by a linear base line. The data are presented as discrete absorbance points that monitor the disappearance of base-on species as the pH is lowered (absorbance at 520 nm, Figure 5a) and the appearance of base-off species (absorbance at 460 nm, Figure 5b). Denaturation of the protein during titration was monitored by a rise in absorbance in the UV region of the spectrum. The extent of this denaturation was compared to a sample denatured with 1 M HCl. At the point in the titration when the spectral changes were near the end point (e.g., at pH values of 2.0), it was found that no more than 25% of the protein had been denatured, as determined by the extent of the UV absorbance increase compared to the denatured control (data not shown).

(D) Photolysis of Methylcobalamin. Photolysis was accomplished using a continuous-wave helium–cadmium Liconix 4240NB laser set at a wavelength of 442 nm. The beam was attenuated with neutral density filters from Newport. The 0.12 cm diameter beam was expanded and collimated to a final diameter of 2.0 cm using KPX205 and KPX076 lenses purchased from Newport. The power of the laser beam at the sample was measured using a Liconix 45PM power meter with a 7 mm diameter sensor probe. A correction for the size of the sensor was required due to the small size of the sensor only being able to measure a fraction of the whole beam. A Gaussian profile was used to approximate the beam power. The general procedures used have been published previously (Chen & Chance, 1993). Typical laser powers were in the range of 0.4 mW. UV–vis spectra were obtained on a Hewlett-Packard 8452A spectrophotometer with a 0.1-s integration time. Buffer conditions and precoating of the cuvette were the same as in the pK_a determination above. Photolysis was

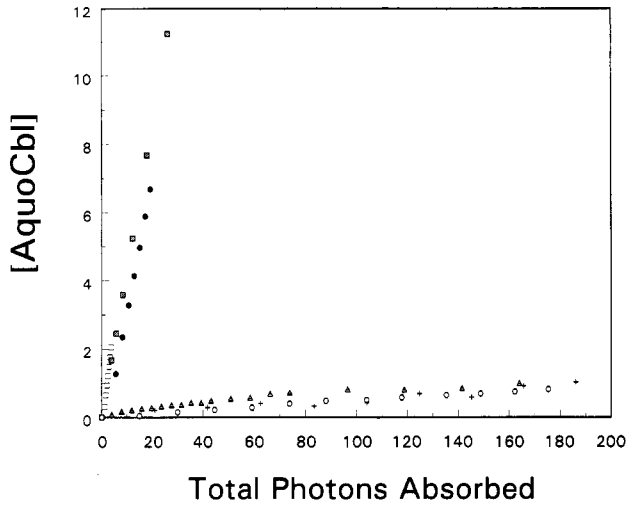

FIGURE 6: Photolysis of free methylcobalamin and methylcobalamin bound to human cobalophilin. The *x*-axis is the concentration of photons absorbed by methylcobalamin (in micromolar), and the *y*-axis is the concentration of aquocobalamin formed (in micromolar; experimental details are in the text). Methylcobalamin data: solid circles, open squares, shaded squares; methylcobalamin–cobalophilin complex data: shaded triangles, crosses, hexagons. The slopes of the lines are equal to the quantum yields of photolysis: 0.35 ± 0.04 for free methylcobalamin data and 0.0055 ± 0.0004 for methylcobalamin–cobalophilin complex data.

accomplished by exposing the sample to the laser beam for intervals from 5 to 30 s, and then an optical spectrum was recorded. The amount of aquocobalamin formed was measured by analyzing the difference spectrum of a partially photolyzed sample compared to that of a fully photolyzed sample. The loss of the methylcobalamin signal exactly matched the appearance of the aquocobalamin signal as the experiments progressed. The photolysis curves were generated by plotting the concentration of aquocobalamin formed versus the (total) concentration of photons absorbed by the methylcobalamin in the sample (Figure 6). The slope of the curve represents the quantum yield of photolysis. Three experiments were performed on free methylcobalamin, and three measurements were performed on the methylcobalamin–cobalophilin complex. These procedures and data analysis methods have been published previously (Chen & Chance, 1993).

RESULTS

The background-subtracted k^3-weighted EXAFS data of human cobalophilin bound to methylcobalamin are shown in Figure 2, and the Fourier transforms of these data are shown in Figure 3. Figure 4 shows the data from Figure 2 compared to a spectrum of free methylcobalamin recorded previously (Sagi & Chance, 1992). The data show little variation above the noise level. Table I shows the results of each chemically reasonable nonlinear least-squares one- or two-atom fit, as well as the results of a three-atom consistency test. $Co(CN)_6$ was used as a model only in the 4/1/1 (three-atom) consistency test, while Co(TPP) was used as the model in all of the other fits. A one-atom type fit was performed in order to provide a context for the improvement in χ^2 (sum of residuals squared) seen in the two-atom fits. The two-atom fits attempted to model the resolved axial ligand distances (5/1 fits) and the average of the four equatorial ligand distances (4/2 fits). These procedures have been described in detail previously (Chance et al., 1986 a–c; Sagi et al., 1990; Sagi & Chance, 1992).

Data were fit with the coordination numbers fixed at six total ligands considering the base-on character of the optical spectra and the overall small spectral shifts seen upon protein binding (Jacobsen et al., 1981). Bond lengths, Debye–Waller factors, and the relative edge energy positions were variables (in the two-atom fits, one of the energies is stepped and one is fixed at a time) for all of the one- and two-atom fits. The three-atom procedure allowed for the determination of the cobalt–upper axial ligand bond distance (cobalt–carbon bond). This distance could not be determined in a 5/1 fit due to its similarity to the cobalt–corrin ring nitrogen distance. The cobalt–upper axial ligand bond distance was allowed to float in the three-atom procedure while two distances provided by the two-atom procedure were held constant (with fixed coordination numbers of 4/1/1) until a valid minimum was found.

Table I shows the results of the one- and two-atom fits along with the three-atom consistency test. The one-atom fit in which the coordination number was held at six ligands gave a very high χ^2 of 25.5. This was expected since all of the bond lengths were averaged together and were treated as cobalt–nitrogen distances. Among the 4/2 two-atom fits, there was only one result that was chemically reasonable, yielding Debye–Waller factos, χ^2 values, edge energy shifts, and bond distances to cobalt that were acceptable. The data show a bond length of 1.90 ± 0.02 Å for the four corrin ring nitrogens (the error is derived from the various analyses discussed in the Materials and Methods section). Figure 4 shows the simulated solution compared to the Fourier-filtered data. The only reasonable 5/1 two-atom fit, where all ligands were fit to a nitrogen model, gave a DMB–cobalt ligand distance of 2.20 ± 0.04 Å. The 4/1/1 consistency test shows that the distance values obtained from the two-atom procedures, when taken together, are consistent with each other and chemically reasonable. In addition, a third distance corresponding to a cobalt–carbon bond of 2.00 Å was required to fit the data.

Figure 5 shows the absorbance changes at 520 and 460 nm upon titration of methylcobalamin bound to cobalophilin with 0.5 M HCl. The raw spectral data show an isosbestic point at 494 nm. This indicates primarily a two-state transition with respect to these spectral bands. The absorbance changes seen in Figure 5 have midpoints for the transition at pH values of 2.7 ± 0.1, which indicates a p*K* value identical to the published value for free methylcobalamin (Hogenkamp, 1974). The titrations shown are not reversible, since by pH 1.0 the protein irreversibly denatures.

Photolyses of Co(III) alkylcobalamins are well-studied reactions where the primary photoproducts are known to be Co(II) and an alkyl radical (Hogenkamp, 1965; Pratt & Whitear, 1971; Taylor et al., 1973; Chen & Chance, 1990, 1993). In the presence of oxygen, the five-coordinate Co(II) species is quickly oxidized to Co(III) and a water ligand binds to cobalt, forming aquocobalamin. Therefore, quantum yields of photolysis for these species can be calculated by measuring the concentration of aquocobalamin produced compared to the concentration of absorbed photons, as described previously (Taylor et al., 1973; Chen & Chance, 1993). The photolysis data of methylcobalamin and methylcobalamin bound to cobalophilin are shown in Figure 6. Data were collected using the same techniques and apparatus for both free and bound methylcobalamin. The data were also treated equally using the same equation and correction factors to calculate the quantum yields (Chen & Chance, 1993). Data are plotted as a function of the (total) concentration of photons absorbed by the methylcobalamin in the sample (abscissa) and the concentration of aquocobalamin photoproduct (ordinate). The quantum yield is the slope of the line: the concentration

of aquocobalamin photoproduct divided by the total photons abosrbed. The quantum yield for free methylcobalamin was determined to be 0.35 ± 0.04, and the quantum yield for methylcobalamin bound to cobalophilin was determined to be 0.0055 ± 0.0004. Essentially, the quantum yield is reduced by a factor of over 60 when protein is bound.

DISCUSSION

The EXAFS results on the methylcobalamin–cobalophilin complex argue against replacement of the DMB ligand by a ligand from the protein. The long Co–N axial distance is characteristic of alkylcobalamins (Rossi et al., 1985; Savage et al., 1987; Sagi et al., 1990; Sagi & Chance, 1992). However, the first-shell EXAFS data only report a nitrogen (or oxygen) ligand at this distance. If a protein ligand had replaced the DMB ligand and was present at 2.20 Å, then the EXAFS first-shell fits would not reveal it. However, steric considerations argue that typical amino acid side chains that are known to bind metal ions in proteins, like histidine, tyrosine, or carboxyl groups, would tend to make a closer approach to the corrin ring than the DMB ligand. However, a recent investigation of the copper site in lactoferrin suggests tyrosine ligands in the range of 2.2–2.8 Å (Smith et al., 1992). Molecular modeling calculations using the methylcobalamin crystal structure illustrate that the hydrogen projecting upward from the benzene ring of the DMB ligand has five nonbonded contact distances of between 2.5 and 3.0 Å with various atoms in the corrin ring (Sagi & Chance, 1992). These repulsions would be absent for all amino acid side chains except tryptophan, which is not known to bind metal ions. However, protein constraints could prevent close approach, so that without more accurate data and higher shell analyses, such a possibility cannot be ruled out. The background-subtracted data for Figure 4 show very little variation for the free methylcobalamin relative to the complex; thus ligand substitution seems unlikely. The titration data provide results similar to those from the EXAFS data, in that the site structure and metal chemistry are similar in the free methylcobalamin and the complex. The pK_a, indicated by the changes in optical spectra, is virtually identical to the value for free methylcobalamin. Thus, the cobalt axial ligand is still weak and protonatable.

The photolysis results are dramatic and, we believe, provide evidence of conformational dynamics in the cobalamin "pocket". There are several possible explanations for reductions in the quantum yield of this magnitude. The first is a change in the photolysis mechanism. In this case, the protein would influence the primary quantum yield (lowering it) and alter the energy-transfer mechanisms from the corrin ring to the cobalt–carbon bond. A second explanation is that the protein influences the rate of geminate recombination, so that the initial bond-breaking event is unchanged but, subsequently, the methyl radical is "caged" and prevented from escaping or reacting with the surrounding protein. A third explanation is that O_2 is prevented from entering the cobalamin pocket, so that oxidation of the Co(II) product is prevented. Any combination of these effects is also possible. The third possibility is unlikely, since considerable evidence indicates that the top axial ligand position is free to react in solution. For example, cyanocobalamin can be formed from aquocobalamin when bound to chicken cobalophilin, while a dicyanide complex is not formed (Marques et al., 1988). The top axial ligand is also accessible to a solid support such as Sephacryl–(aminopropyl)cobalamin for affinity purification of the protein (Allen et al., 1974). The success of cobalamin affinity techniques in isolating cobalophilin confirms that the top axial ligand position could not be protected from oxygen diffusion by the protein.

Thermolysis of alkylcobalamins is known to produce the same initial products as photolysis, namely, an alkyl radical and Co(II). A number of alkylcobalamins have been investigated by this technique in order to determine bond dissociation energies (Finke & Hay, 1984; Geno & Halpern, 1987; Hay & Finke, 1987). When alkylcobalamins were bound to cobalophilin from chicken, their thermal decomposition was inhibited, i.e., the proteins protect the cobalt–carbon bond from homolytic fission. The stabilization was on the order of 3.3–4.3 kcal mol^{-1} (275–1400-fold) compared with the free alkylcobalamin decomposition (Brown et al., 1991). The authors suggest that the protein stabilizes the base-on form of the alkylcobalamins studied by lowering the activation entropy of homolysis of the cobalt–upper axial ligand bond. In essence, the effect is not thought of as pure (enthalpic) bond strengthening. These results are essentially in agreement with ours, and they suggest that explanation 1 above should, at present, be rejected as a working hypothesis. It is unlikely that the protein influences the thermolytic and photolytic primary dissociation processes in the same way, since the photolytic and thermolytic bond-cleavage mechanisms are unlikely to be similar. Explanation 2, which suggests that the protein influences the geminate recombination rate (increasing it dramatically), could be observed as a reduction in both thermolytic and photolytic cleavage when protein is bound. The mechanism whereby the protein might increase geminate recombination is currently a mystery. To do so, in order to cage a reactive and mobile methyl radical, a conformational change must occur upon cobalt–carbon bond cleavage that closes up the cobalamin pocket to increase the extent of geminate recombination. This is an area for future study.

It is well-known that cobalamins bound to cobalophilin in circulation have an extremely long half-life (Schneider & Stroinski, 1987). It appears that one function of cobalophilin may be to protect alkylcobalamins *in vivo* from light that penetrates into blood vessels, capillaries, and the lymph system. The effects of light on skin and other organs have been studied (Wilson et al., 1984; Wan et al., 1981a,b). A significant quantity of light penetrates, with the depth of penetration increasing steadily within the wavelength region 600–800 nm (Wilson et al., 1984). In order to explore the possibility that the protective effect of the protein is physiologically relevant, we present calculations on the effective dosage received *in vivo* and compare the numbers to the typical dosages utilized in phototherapy for jaundiced babies. The definition of effective absorbed light dose is given by Profio et al. (1984):

$$D = (a/p)CWKt$$

where the effective dose (D) is expressed in joules/kilogram. The extinction coefficient, a, is expressed in m^{-1} $(\mu g/g)^{-1}$. This was calculated for the methylcobalamin and bilirubin spectra at 2-nm intervals from 200 to 600 nm. The bilirubin spectrum was taken from McDonagh (1979). The tissue density, p, was taken as 1030 kg m^{-3} (Profio et al., 1984), and the concentration (C) of the light-sensitive complex was 5.5 × 10^{-4} $\mu g/g$ for the methylcobalamin–protein complex (Schneider and Stroinski, 1987) and either 135 $\mu g/g$ for bilirubin in a jaundiced baby or 7 $\mu g/g$ for a healthy baby (Donzelli, 1988). The power incident (W) for the methylcobalamin–protein calculation was based on the sun's irradience curve from 200 to 600 nm in W m^{-2}, again in 2-nm intervals to correspond to the extinction coefficients (Weast,

1982); for the bilirubin calculations it was based on the irradience from a typical hospital phototherapy setup (Christensen et al., 1988). The quantum yield (K) for bilirubin in blood was taken to be 0.0036 (Ennever, 1988); those for free and protein-bound methylcobalamin were as above. The time (t) of light exposure was assumed to be 12 h (43 200 s).

The effective dose for a typical jaundiced baby according to the above is 16 597 J/kg; for a 4-kg baby this amounts to 66 389 J. A healthy baby has a 12-h dose of 851 J/kg or 3404 J. In comparison, the absorbed dose for a normal adult (80 kg) would be 10 480 J (131 J/kg) in the absence of cobalophilin protection. The actual situation is a scant 176 J (2.2 J/kg) due to the lowered quantum yield in the presence of protein. These numbers provide some context for the physiological relevance of the protection from light. Twelve hours of phototherapy is generally enough to provide substantial clearance of bilirubin in addition to the normal clearance mechanisms. Substantial sun exposure would provide an alarmingly high absorbed dose for methylcobalamin in the absence of cobalophilin protection. Unprotected cobalamins would be easily photolyzed to aquocobalamin, and the coenzyme forms would need to be resynthesized frequently. Therefore, cobalophilin may be useful in precluding this photolysis process and in preventing the wasteful resynthesis of coenzyme forms of B_{12}.

REFERENCES

Allen, R. H., Burger, R. L., Mehlman, C. S., & Majerus, P. W. (1974) *Methods Enzymol. 34*, 305.

Andrews, E. R., Pratt, J. M., & Brown, K. L. (1991) *FEBS Lett. 281*, 90.

Brown, K. L., Marques, H. M., & Jacobsen, D. W. (1988) *J. Biol. Chem. 263*, 1872.

Brown, K. L., Brooks, H. B., Behnke, D., & Jacobsen, D. W. (1991) *J. Biol. Chem. 266*, 6737.

Chance, M. R., Powers, L., Kumar, C., & Chance, B. (1986a) *Biochemistry 25*, 1259.

Chance, M. R., Powers, L., Poulos, T., & Chance, B. (1986b) *Biochemistry 25*, 1266.

Chance, M. R., Parkhurst, L., Powers, L., & Chance, B. (1986c) *J. Biol. Chem. 261*, 5689.

Chen, E., & Chance, M. R. (1990) *J. Biol. Chem. 265*, 12987.

Chen, E., & Chance, M. R. (1993) *Biochemistry 32*, 1480.

Christensen, T., Stottum, A., Brunborg, G., & Reitan, J. F. (1988) in *Light in Biology and Medicine, Vol. 1* (Douglas, R. H., Moan, J., & Dall'Acqua, F., Eds.) pp 153–159, Plenum Publishers, New York.

Cramer, S. P.; Tench, O., Yocum, M., & George, G. N. (1988) *Nucl. Instrum. Methods A266*, 586.

Davies, G., & Stark, G. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 651.

Donzelli, G. P. (1988) in *Light in Biology and Medicine, Vol. 1* (Douglas, R. H., Moan, J., & Dall'Acqua, F., Eds.) pp 161–171, Plenum Publishers, New York.

Elsenhans, B., & Rosenberg, I. H. (1984) *Biochem. J. 23*, 805.

Ennever, J. F. (1988) in *Light in Biology and Medicine, Vol. 1* (Douglas, R. H., Moan, J., & Dall'Acqua, F., Eds.) pp 143–151, Plenum Publishers, New York.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606.

Finke, R. G., & Hay, B. P. (1984) *Inorg. Chem. 23*, 3043.

Frisbie, S. M. (1992) Ph.D. Dissertation, Georgetown University, Washington, D.C.

Geno, M. K., & Halpern, J. (1987) *J. Am. Chem. Soc. 109*, 1238.

Gilbert, H. S. (1974) *Blood 44*, 926.

Gullberg, R. (1976) *Scand. J. Gastroenterol. 11*, 225.

Hall, C. A., & Finkler, A. E. (1971) *Methods Enzymol. 18*, 108.

Hay, B. P., & Finke, R. G. (1987) *J. Am. Chem. Soc. 109*, 8012.

Hippe, E., & Olesen, H. (1971) *Biochim. Biophys. Acta 243*, 83.

Hippe, E., Haber, E., & Olesen, H. (1971) *Biochim. Biophys. Acta 243*, 75.

Hogenkamp, H. P. C. (1974) in *Cobalamin-Biochemistry and Pathophysiology* (Babior, B. M., Ed.) p 21, John Wiley and Sons, New York.

Hogenkamp, H. P. C., Rush, J., & Swenson, C. (1965) *J. Biol. Chem. 240*, 3641.

Jacobsen, D. W., & Huennekens, F. M. (1986) *Methods Enzymol. 123*, 28.

Jacobsen, D. W., Montejano, Y. D., & Huennekens, F. M. (1981) *Anal. Biochem. 113*, 164.

Johnston, J., Bollekens, J., Allen, R. H., & Berliner, N. (1989) *J. Biol. Chem. 264*, 15754.

Kruger, G. (1978) *Acta Crystallogr. B346*, 915.

Lee, P., Citrin, P., Eisenberger, P., & Kincaid, B. (1981) *Rev. Mod. Phys. 53*, 769.

Lytle, F., Sayers, D., & Stern, E. (1989) *Physica B, 158*, 701.

Madura, P., & Scheidt, W. R. (1978) *Inorg. Chem. 15*, 3182.

Marques, H. M., Brown, K. L., & Jacobsen, D. W. (1988) *J. Biol. Chem. 263*, 12378.

McDonagh, A. F. (1979) in *The Porphyrins: Biochemistry, Part A, Vol. 1* (Dolphin, D., Ed.) pp 385–389, Academic Press, New York.

McGilvery, R. W., & Goldstein, G. W. (1983) *Biochemistry: A Functional Approach*, 3rd ed., p 670, W. B. Saunders, Philadelphia, PA.

Nexo, E. (1978) *Scand. J. Haematol. 20*, 221.

Nexo, E., & Olesen, H. (1982) in *B_{12}: Biochemistry and Medicine* (Dolphin, D., Ed.) Vol. 2, pp 57–84, Wiley and Sons, New York.

Powers, L., Chance, B., Ching, Y., & Angiolillo, P. (1981) *Biophys. J. 34*, 465.

Pratt, J. M., & Whitear, B. R. D. (1971) *J. Chem. Soc. (A)*, 252.

Profio, A. E., Wudl, L. R., & Sarnaik, J. (1984) in *Advances in Experimental Medicine and Biology, Vol. 193* (Kessel, D., Ed.) pp 35–41, Plenum Press, New York.

Rossi, M., Glusker, J. P., Randaccio, L., Summers, M. F., Toscano, P. J., & Marzilli, L. G. (1985) *J. Am. Chem. Soc. 107*, 1729.

Sagi, I., & Chance, M. R. (1992) *J. Am. Chem. Soc. 114*, 8061.

Sagi, I., Wirt, M. D., Chen, E., Frisbie, S. M., & Chance, M. R. (1990) *J. Am. Chem. Soc. 112*, 8639.

Savage, H., Lindley, P., Finney, J., & Timmins, P. (1987) *Acta Crystallogr. B43*, 280.

Schneider, Z., & Stroinski, A. (1987) *Comprehensive B_{12}*, W. de Gruyter, Berlin.

Smith, C. A., Anderson, B. F., Baker, H. M., & Baker, E. N. (1992) *Biochemistry 31*, 4527.

Taylor, R., Smucker, L., Hanna, M. L., & Gill, J. (1973) *Arch. Biochem. Biophys. 156*, 521.

Wan, S., Anderson, R. R., & Parrish, J. A. (1981a) *Photochem. Photobiol. 34*, 493.

Wan, S., Parrish, J. A., Anderson, R. R. & Madden, M. (1981b) *Photochem. Photobiol. 34*, 679.

Weast, R. (1982) in *CRC Handbook of Chemistry and Physics*, CRC Press, Boca Raton, FL.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Wilson, B. C., Jeeves, W. P., Lowe, D. M., & Adam, G. (1984) Light Propagation in Animal Tissues in the Wavelength Range 375–825 Nanometers. in *Porphyrin Localization and Treatment of Tumors*, p 115, Alan R. Liss, Inc., New York.